Fate of [^{14}C]Emamectin Benzoate in Cabbage. 2. Unextractable Residues

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Cabbage plants were treated with seven weekly applications of [^{14}C]emamectin benzoate followed by a final application of [^{14}C/^{3}H]emamectin benzoate at two rates; the maximum expected commercial use rate of 0.015 lbs a.i./acre (1×) or an exaggerated rate of 0.075 lbs a.i./acre (5×). Methanol homogenates of cabbage heads were extracted with methanol/water containing ammonium acetate. Approximately 14−26%, or 58−95 ppb for 1× plants and 188−492 ppb for 5× plants, of the total residues remained unextractable. The unextractable residues were characterized by serial treatment with buffer, cellulase, α-amylase, pectinase, and protease, solubilization with hot dimethyl sulfoxide (DMSO), and reflux with 0.5 N sodium hydroxide (NaOH). A portion of the amylase hydrolysate was treated with maltase. All samples, including final uncharacterized residues, were analyzed for radioactivity. Selected fractions were assayed for glucose and protein. Generally, recovered radioactivity was released in the following order: protease (23−29%) > DMSO (15−29%) > NaOH (12−18%) > cellulase (9−17%) > buffer (9−16%) > α-amylase (7−9%) > pectinase (3−5%). Only about 1% of the initial unextractable residues remained uncharacterized after the procedure. The results suggest incorporation of radioactivity into natural products such as protein, cellulose, lignin, and starch.

Keywords: *Emamectin benzoate; unextractable; natural products*

INTRODUCTION

The avermectins are macrolides produced by the actinomycete *Streptomyces avermitilis*. Abamectin, consisting of the natural avermectins B_{1a} and B_{1b}, is currently used worldwide as an insecticide. Emamectin benzoate (MK0244, Figure 1), a synthetic derivative of abamectin, is 4″-deoxy-4″-(*epi*-methylamino)avermectin B_1 (MAB1) and is a mixture of two active compounds: 4″-deoxy-4″-(*epi*-methylamino)avermectin B_{1a} (MAB1A) and 4″-deoxy-4″-(*epi*-methylamino)avermectin B_{1b} (MAB1B). The homologue ratio composition of emamectin benzoate is greater than 9 B_{1a}:1 B_{1b}. Emamectin benzoate, applied at very low use rates to crops, is an effective insecticide against lepidopteran larvae.

A metabolism study in cabbage was performed using radiolabeled MAB1A since the MAB1A homologue is the major constituent of emamectin benzoate. Investigations of the extractable residues found in this study were the subject of a separate report (Crouch et al., 1997). Here, we report on studies of the residues that were not extractable with methanol/water containing ammonium acetate. The present study of the unextractable residues in MAB1A-treated cabbage was the first time enzymes, specific for natural products, have been used to characterize residues in avermectin-treated plants. The unextractable residues, if not related to natural products, could be of toxicological concern since they constituted up to 26% of the total ^{14}C-residue.

MATERIALS AND METHODS

Test Compounds, Cabbage Cultivation and Treatment, Specimen Processing, and Extraction of Radioactivity. Radiolabeled MAB1A, emulsifiable concentrate formulation (EC), and additional surfactant were supplied by Merck Research Labs. All specimens of cabbage, consisting of wrapper leaf plus head, came from plants treated at 0.015 lbs a.i./acre (1×) or a 5× exaggerated rate. Plants were harvested at 2 h, 1, 3, 7, or 10 day preharvest intervals (PHI). All plants were treated with seven weekly applications of [^{14}C]-MAB1A followed by a single application of [^{14}C/^{3}H]MAB1A. Specimens of cabbage were prepared as described in Crouch et al., 1997.

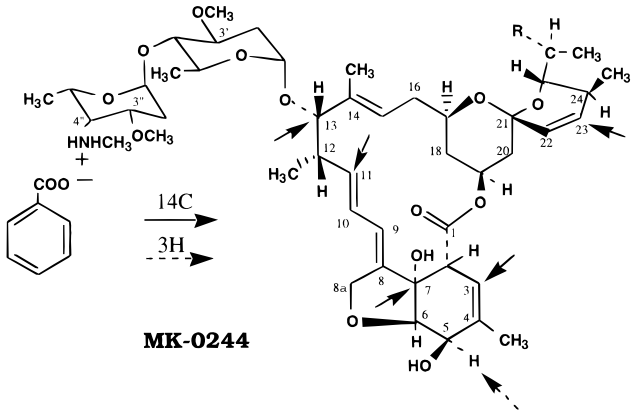

Figure 1. Structure of major (4″-deoxy-4″-(*epi*-methylamino)-avermectin B_{1a}, MAB1A) and minor (4″-deoxy-4″-(*epi*-methylamino)avermectin $B1_b$, MAB1B) homologues of MK-0244.

For unextractable residues, subsamples of minced cabbage from three plants per PHI and rate were composited (10 samples in all). They were homogenized with approximately 2 mL of methanol per gram of minced cabbage using a Brinkmann Model 10/35 Polytron. Total wet weights of approximately 80−150 g of minced cabbage for each of the 10 samples were used. Approximately 230−420 g of methanol homogenate was obtained for the composite samples.

The homogenates were first extracted with methanol/water (containing ammonium acetate) to remove the extractable

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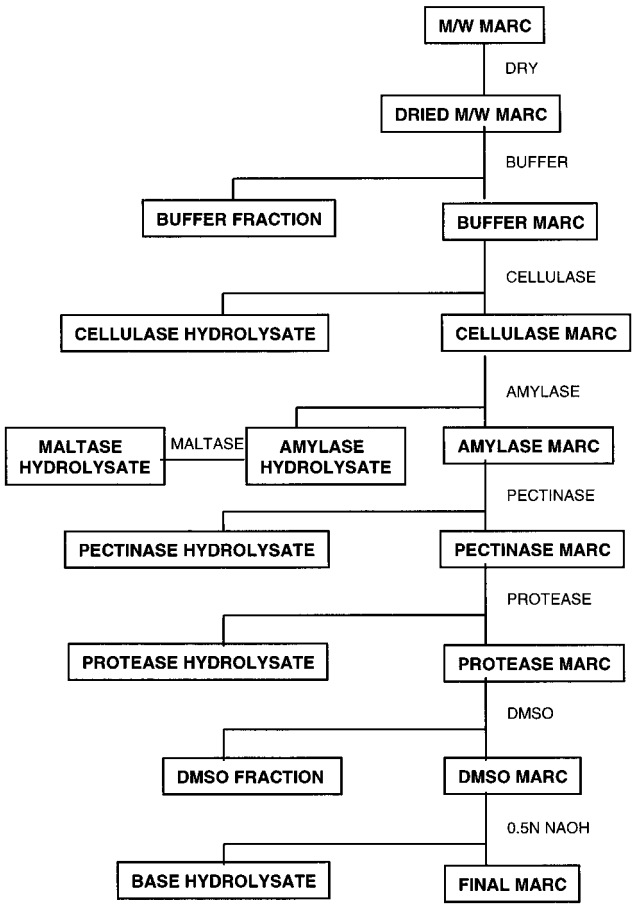

Figure 2. Marc remaining after M/W extraction of cabbage was dried, ground, and subjected serially to buffer, enzymolyses (cellulase, α-amylase, pectinase, protease), hot DMSO, and reflux with base. See Materials and Methods for details.

residues as described previously (Crouch et al., 1997). The marcs or material remaining after extraction with methanol/water/ammonium acetate (M/W marcs) contained the unextractable residues. They were dried for several days under vacuum at approximately 35 °C in a Fisher Model 280 vacuum oven. After drying, the M/W marcs were ground to a powder with a mortar and pestle or Waring blender.

Serial Extraction of M/W Marc with Solvents of Increasing Polarity. Approximately 100 mg of the dried M/W marc from the composited 5× plants (3 days PHI) were extracted serially with 3 mL each of solvents of increasing polarity in the following order: cyclohexane, toluene, chloroform, water, and finally 2% triton X-100 detergent in water. The sample extractions were centrifuged, and the supernatants were removed and assayed for radioactivity by liquid scintillation counting (LSC).

Fractionation of Methanol/Water−Unextractable Residue. Portions of the dried M/W marcs from the 1× and 5× plants at all PHI's were then subjected to a multistep procedure consisting of suspension in buffer, enzymolysis, DMSO solubilization, and base hydrolysis as described in detail below. See Figure 2 for a schematic outline of the fractionation of these unextractable residues. Also, one control sample containing no M/W marc was taken through the procedure to correct for any contributions of the enzymes used to the natural products assayed for in the unextractable residue fractions.

The order of the enzymolysis/extraction steps shown in Figure 2 was determined by a series of probe experiments. In particular, the placement of enzyme incubations was established by incubation of dried cabbage M/W marcs with enzymes separately and in various combinations. The procedure in Figure 2 was utilized because it resulted in maximum release of radioactivity. Especially important was the placement of the protease step after the steps involving solubilization of the cell wall. Separate experiments with various proteases were also performed to optimize release of radioactivity. Crude bovine pancreas protease was chosen on the basis of these studies.

Step 1: Buffer Fraction. An aliquot (approximately 1 g) of each dried M/W marc was dissolved in 200 mL of sodium acetate buffer (50 mM, pH 5) in a shaking water bath (approximately 37 °C) for 1 h. The buffer fraction was then centrifuged at 2250 rpm on an IEC Model UV centrifuge for 10 min. The resultant supernatant was removed, aliquots were taken for radioactivity analysis, and the remainder was stored frozen. The pellet (buffer marc) was then subjected to cellulase digestion. This buffer step served to remove any radioactivity that would be removed by buffer alone in the first enzymolysis step (cellulase digestion).

Step 2: Cellulase Digestion. The buffer marc was resuspended at a concentration of 5 mg equiv (weight based on the initial specific activity of MAB1A) of dried M/W marc/mL in 200 mL of sodium acetate buffer (50 mM, pH 5) containing 10 U/mL cellulase (*P. funiculosum*, Sigma). The cellulase digestion was performed in a shaking water bath (approximately 37 °C) for 2.5 h. The cellulase-incubated mixture was then centrifuged at 2250 RPM on an IEC Model UV centrifuge for 10 min. The resultant supernatant (cellulase hydrolysate) was removed, aliquots were taken for radioactivity analysis, and the remainder was stored frozen. The pellet (cellulase marc) was stored frozen overnight and subjected to amylase digestion the next day.

Step 3: Amylase Digestion. The cellulase marc was resuspended at a concentration of 5 mg equiv of dried M/W marc/mL in 200 mL of potassium phosphate buffer (50 mM, pH 6.9) containing 10 U/mL of α-amylase (*Aspergillus oryzae*, Sigma). The amylase incubation was performed in a shaking water bath (approximately 37 °C) for 2.5 h. The amylase digestion was then centrifuged at 2250 rpm on an IEC Model UV centrifuge for 10 min. The resultant supernatant (amylase hydrolysate) was removed, aliquots were taken for radioactivity analysis, and the remainder was stored frozen. The pellet (amylase marc) was then subjected to pectinase digestion. A portion of the amylase hydrolysate was thawed and incubated with maltase (Sigma, Bakers' yeast).

Step 4: Pectinase Digestion. The amylase marc was resuspended at a concentration of 5 mg equiv of dried M/W marc/mL in 200 mL of sodium acetate buffer (50 mM, pH 4.0) containing 10 U/mL pectinase (*Aspergillus niger*, Sigma). The pectinase digestion was performed in a shaking water bath (approximately 37 °C) for 2.5 h. The amylase incubated mixture was then centrifuged at 2250 rpm on an IEC Model UV centrifuge for 10 min. The resultant supernatant (pectinase hydrolysate) was removed, aliquots were taken for radioactivity analysis, and the remainder was stored frozen. The pellet (pectinase marc) was stored frozen for 4 days and then was subjected to protease digestion.

Step 5: Protease Digestion. The pectinase marc was resuspended at a concentration of 5 mg of dried M/W marc equiv/mL in 200 mL of potassium phosphate buffer (50 mM, pH 7.5) containing 10 U/mL of protease (crude bovine pancreas, Sigma). The protease digestion was performed in a shaking water bath (approximately 37 °C) for 2.5 h. The protease-incubated mixture was then centrifuged at 2250 rpm on an IEC Model UV centrifuge for 10 min. The resultant supernatant (protease hydrolysate) was removed, aliquots were taken for radioactivity analysis, and the remainder was stored frozen. The pellet (protease marc) was stored on ice until extraction with DMSO on the same day.

Step 6: DMSO Solubilization. The protease marc was resuspended in DMSO at a concentration of 5 mg of dried M/W marc equiv/mL in 200 mL of DMSO. The mixture was then extracted overnight at approximately 80 °C on a hot plate with stirring. The DMSO extract was cooled and then centrifuged at 2250 rpm on an IEC Model UV centrifuge for 20 min. The resultant supernatant (DMSO fraction) was removed, aliquots were taken for radioactivity analysis, and the remainder was stored in a refrigerator overnight. The pellet (DMSO marc) was stored in a freezer until hydrolysis with base the next day.

Step 7: Base Hydrolysis. The DMSO marc was resuspended in 0.5 N sodium hydroxide (NaOH) at a concentration of 10 mg of dried M/W marc equiv/mL in a total volume of 100 mL.

The solution was refluxed for 2 h and then cooled. The base hydrolysis solution was first decanted after settling, leaving a precipitate with some solution. The remaining solution plus precipitate was then centrifuged at 2500 rpm on an IEC Model HNS II centrifuge for 10 min. The resultant supernatant was removed and combined with the decanted solution to yield the base hydrolysate. Aliquots were taken for radioactivity analysis, and the remainder was stored at less than −10 °C. The pellet (final marc) was suspended in methanol and stored below −10 °C.

The M/W marcs containing the total unextractable residues and selected fractions resulting from the enzymatic, DMSO solvent extraction and base hydrolysis treatment of the M/W marcs were assayed for two natural products, protein and glucose as described below. These natural products were expected to be released from the M/W marcs during the multistep procedure (Figure 2).

Natural Product Characterization and Quantitation. For protein analysis (Lowry et al., 1951) portions (approximately 15 mg) of the dried 5× M/W marcs were homogenized in 10 mL of water using a glass homogenizer with Teflon pestle. For the M/W marc homogenates (100−400 μL), base hydrolysates (100−400 μL), final marcs suspended in methanol (100−400 μL), DMSO extracts (20−90 μL), protease hydrolysates (20−90 μL), and buffer fractions (100−400 μL) duplicate aliquots of several volumes within the ranges indicated were assayed. Similar volumes were used for the control samples obtained using the procedure in the absence of the M/W marc. Protein analysis was not performed on the amylase, pectinase, and cellulase hydrolysates. For the protein assay all sample aliquots and bovine serum albumin (BSA, Sigma) standards were diluted to a volume of 0.5 mL with water, and the total volume after addition of reagents was 2.7 mL. The standard curve samples contained 0−200 mg of BSA per tube. The absorbance of the samples and standards versus water at 750 nm was obtained, using a Perkin-Elmer Model 320 spectrophotometer, after background absorbance was subtracted out.

Selected fractions from the 5× M/W marcs were assayed for glucose by the Trinder method (Trinder, 1969) using a Sigma reagent kit. Duplicate aliquots of several volumes (20−100 μL) of the appropriate controls for each fraction and the buffer fractions, cellulase hydrolysates, amylase hydrolysates, pectinase hydrolysates, protease hydrolysates, and the maltase incubations of the amylase hydrolysates were diluted to 100 μL with water for assay. A standard curve with tubes containing 0−70 μg in 100 μL water was also prepared. A volume of 1.5 mL of the reagent prepared according to the kit instructions was added to each tube. The absorbance at 505 nm was read versus water after incubation at room temperature for at least 2 h.

For characterization of the amylase fractions, an incubation with α-glucosidase (maltase) was performed. Maltase was added to aliquots (2 mL) of the amylase hydrolysates and the appropriate control to give a concentration of approximately 10 U/mL. The enzyme incubation was then carried out at 37 °C for 2.5 h in a shaking water bath. After 2.5 h, the maltase incubations were then stored in a freezer.

RESULTS

Methanol/Water Extraction. All cabbage plants were treated with one application of [^{14}C/^{3}H]MAB1A as the last of eight weekly applications. However, due to the very low levels of tritium in the unextractable residues (not shown), only data for ^{14}C are presented. Table 1 shows the percentages and ppb concentrations of the recovered total radioactive residue and M/W-extractable and M/W-unextractable residues. The percentages of extractable and unextractable residues for the 1×- and 5×-treated plants were comparable at each PHI. From 2 h to 10 days, after the last of eight weekly applications, the percentage of unextractable residue ranged from 20 to 25% for the 1× treated plants and from 14 to 22% for the 5× treated plants. The concentration of M/W-unextractable ^{14}C-residue (expressed as MAB1A equivalents) was rather constant at about 80−100 ppb in the 1× plants through 7 days PHI and then declined to about 60 ppb by 10 days PHI (Table 1). The concentration of unextractable ^{14}C-residue (expressed as MAB1A equivalents) was rather constant at about 500 ppb in the 5× plants through 3 days PHI and then declined to less than 300 ppb over the next two PHIs of 7 and 10 days. The values for total radioactive residue and percentages of residues extractable and unextractable for composited cabbage plants (Table 1) were similar to those obtained for the mean values from the same individual cabbage plants (Crouch et al., 1997).

Table 1. Total, M/W-Extractable, and M/W-Unextractable ^{14}C Residue of Composited 1× and 5× Plants[a]

	% of TRR[b]			MAB1A equiv[c]		
PHI	ER[c]	UR[d]	total	TRR (ppb)	ER (ppb)	UR (ppb)
1×						
2 h	80.49	19.51	100	488	393	95
1 day	77.49	22.51	100	410	317	92
3 days	74.48	25.52	100	342	254	87
7 days	75.29	24.71	100	331	249	82
10 days	74.82	25.18	100	230	172	58
5×						
2 h	85.84	14.16	100	3475	2983	492
1 day	82.28	17.72	100	2588	2129	459
3 days	79.75	20.25	100	2424	1933	491
7 days	78.22	21.78	100	863	675	188
10 days	77.79	22.21	100	1219	949	271

[a] The composited tissue of three plants from each PHI and rate was extracted with methanol/water containing ammonium acetate as previously described (Crouch and Feely, 1995). Recoveries ranged from 90 to 114%. The extractable and unextractable residues were expressed as ppb MAB1A equivalents. [b] Total radioactive residue (TRR) in cabbage; values are normalized for recovered radioactivity. [c] Extractable residue. [d] Unextractable residue.

Fractionation of Methanol/Water-Unextractable Residue. The M/W marc from 5× plants at 3 days PHI was extracted serially with 3 mL each of solvents of increasing polarity in the order: cyclohexane, toluene, chloroform, water, and finally 2% triton X-100 detergent in water. Less than 9% of the total radioactivity in the M/W marc was extracted by this procedure (data not shown). In contrast, using the fractionation procedure for unextractable residues shown in Figure 2, approximately 99% of the total unextractable radioactivity was released. The proportion of ^{14}C radioactivity released during each step of the procedure remained rather constant over the range of PHIs (Table 2), although the total unextractable residue declined significantly by 7 (5×) or 10 (1×) days PHI (Table 1). The percentages of recovered ^{14}C radioactivity released from the M/W marcs was generally of the following order: protease hydrolysate (23−29%) > DMSO fraction (15−29%) > base hydrolysate (12−18%) > cellulase hydrolysate (9−17%) > buffer fraction (9−16%)> amylase hydrolysate (7−9%) > pectinase hydrolysate (3−5%) (Table 2).

Natural Product Characterization and Quantitation. The M/W marcs from the cabbage of the 5× plants and the buffer and DMSO fractions and protease and base hydrolysate fractions of the M/W marcs (Figure 2) were assayed for protein content (Table 3). The M/W marcs contained approximately 70% of the cabbage protein (data not shown). The percentage of protein in the assayed fractions of the unextractable residues remained relatively constant regardless of PHI. The mean percentage of protein recovered from the M/W marc fractions for all PHIs was approximately 57% for

Table 2. Distribution of Radioactivity in Unextractable Residue Fractions from 1× and 5× plants[a]

	% ^{14}C radioactivity)								
PHI	BF	CH	AH	PEH	PRH	DF	BH	FM	total
				1× Plants					
2 h	12.70	12.08	6.10	2.56	25.23	17.55	10.20	0.68	87.11[b]
	14.58	*13.87*	*7.01*	*2.94*	*28.96*	*20.14*	*11.71*	*0.78*	*100*[c]
1 day	12.42	11.43	6.51	3.90	21.08	18.47	11.08	0.60	85.49
	14.52	*13.37*	*7.62*	*4.56*	*24.66*	*21.60*	*12.96*	*0.70*	*100*
3 days	10.75	11.93	7.57	3.93	23.11	17.12	13.40	0.94	88.75
	12.12	*13.44*	*8.53*	*4.439*	*26.04*	*19.29*	*15.10*	*1.05*	*100*
7 days	14.41	15.15	6.67	3.71	23.49	15.73	11.25	1.05	91.47
	15.75	*16.56*	*7.29*	*4.06*	*25.68*	*17.20*	*12.30*	*1.15*	*100*
10 days	13.56	14.70	7.49	4.97	25.36	13.74	11.36	1.11	92.28
	14.69	*15.93*	*8.12*	*5.38*	*27.48*	*14.889*	*12.31*	*1.20*	*100*
				5× Plants					
2 h	8.53	7.84	6.55	3.52	22.11	26.33	16.30	0.72	91.88[b]
	9.28	*8.53*	*7.13*	*3.83*	*24.06*	*28.65*	*17.73*	*0.79*	*100*[c]
1 day	14.68	9.66	7.30	2.87	21.60	22.96	14.49	0.69	94.24
	15.57	*10.25*	*7.75*	*3.05*	*22.92*	*24.36*	*15.37*	*0.73*	*100*
3 days	11.46	8.49	6.40	2.16	21.87	19.53	15.21	0.65	85.79
	13.36	*9.90*	*7.46*	*2.52*	*25.50*	*22.77*	*17.73*	*0.76*	*100*
7 days	14.34	10.73	7.78	3.71	23.79	16.86	12.79	1.03	90.45
	15.85	*11.86*	*8.60*	*4.10*	*26.30*	*18.64*	*14.14*	*1.13*	*101*
10 days	11.09	9.23	7.58	3.30	22.74	15.90	15.59	0.82	86.26
	12.86	*10.69*	*8.79*	*3.82*	*26.37*	*18.44*	*18.08*	*0.95*	*100*

[a] The residues in the M/W marc from composited plants (3 plants per PHI) were fractionated by a serial enzymatic/solubilization/base hydrolysis procedure (Figure 2), and the residue released by each step and that remaining in the final marc was determined. The radioactivity is expressed as percent of total ^{14}C radioactivity in the M/W marc. Abbreviations for column headings are as follows: BF, buffer fraction; CH, cellulase hydrolysate; AH, amylase hydrolysate; PEH, pectinase hydrolysate; PRH, protease hydrolysate; DF, DMSO fraction; BH, base hydrolysate; FM, final marc. [b] Percent of M/W marc. [c] Italicized numbers are percent of recovered radioactivity.

Table 3. Percent Protein Content of Unextractable Residue Fractions from M/W Marcs of 5× Plants[a]

	fraction				
PHI	BF	PRH	DF	BH	FM
2 h	8.38	61.74	23.23	6.50	0.27
1 day	13.78	53.86	24.19	7.81	0.36
3 days	11.11	56.46	23.79	8.25	0.39
7 days	11.60	54.17	23.85	9.89	0.49
10 days	9.40	58.92	23.90	7.40	0.37
mean	10.85	57.03	23.79	7.97	0.38
SD	2.09	3.33	0.35	1.25	0.08

[a] The total percentage protein content of the fractions of unextractable residues obtained by the serial enzymolysis/extraction/hydrolysis procedure was determined by the method of Lowry et al. (1951). The results were expressed as the percent of the total protein recovered in each fraction of the M/W marcs: BF, buffer fraction; PRH, protease hydrolysate; DF, DMSO fraction; BH, base hydrolysate; FM, final marc. Recoveries of protein from M/W marcs ranged from 93 to 120%.

the protease hydrolysates, 24% for the DMSO fractions, 11% for the buffer fractions, 8% for the base hydrolysates, and less than 1% in the final marcs (Table 3). The remaining unextractable residue fractions, which were derived by enzymolysis, were not assayed for protein since the amount of added protein was expected to be far larger than any protein released. However, the absorbance values for the protease hydrolysates in the protein assay were approximately 1.5-fold that of the protease alone at the protease concentration used in the procedure. Appropriate blanks were subtracted from sample absorbances for the protease hydrolysates. Attempts to analyze the protease hydrolysates that had been digested with acid and derivatized with dansyl chloride by HPLC were unsuccessful due to low amounts of radioactivity.

The glucose content of several of the unextractable residue fraction types from 5× plants was determined (Table 4). The glucose content of the amylase hydrolysates was determined before (not shown) and after incubation with maltase. The cellulase, pectinase, maltase-treated amylase, and protease hydrolysates accounted for a mean of approximately 72%, 16%, 9%, and 3%, respectively, of the total glucose released for all PHIs; no glucose was found in the buffer fractions (Table 4). Although there was some variability in the percent distribution of glucose among the unextractable residue fractions, no trends were apparent (Table 4). However, the released glucose expressed as its concentration in the dried M/W marcs was rather constant at approximately 170 mg/g (Table 4). On the basis of total released radioactivity and glucose in the cellulase hydrolysates, the specific activity of glucose would be about 1 dpm/μg versus 29 μCi/mg for applied MAB1a (Crouch et al., 1997) or about 64 000 dpm/μg. This specific activity for glucose would be a maximum value since the cellulase hydrolysate was not analyzed for other components. The other unextractable residue fraction types (base hydrolysates, DMSO fractions, final marcs) were not assayed for glucose.

Table 4. Percent Glucose Content of Unextractable Residue Fractions and Glucose Concentration from 5× Plants[a]

	fraction					
PHI	BF	CH	AH+ maltase	PEH	PRH	mg/g[b]
2 h	0	66.0	8.2	21.9	3.9	171.5
1 day	0	80.3	7.5	9.7	2.6	154.8
3 days	0	83.1	8.6	6.1	2.3	163.1
7 days	0	69.5	9.9	17.0	3.6	178.7
10 days	0	59.7	12.5	24.2	3.6	187.2
mean	0	71.7	9.3	15.8	3.2	171.1
SD	0	9.8	2.0	7.8	0.7	10.3

[a] The total glucose content of various fractions of the unextractable residues obtained from aliquots of the M/W marcs from the composited 5× plants was determined by the Trinder method. The results were expressed as the percent of the total glucose recovered in each fraction of the M/W marcs. The amylase hydrolysate was treated with maltase and reanalyzed for glucose: BF, buffer fraction; CH, cellulase hydrolysate; AH, amylase hydrolysate; PEH, pectinase hydrolysate; PRH, protease hydrolysate. [b] mg glucose per g dry weight of M/W marc.

DISCUSSION

Avermectins, such as abamectin and emamectin benzoate, degrade rapidly on glass in the presence of light (Crouch et al., 1991; Feely et al., 1992) to a complex mixture of residues. This rapid degradation also happens on plants where loss of total radioactive residue and incorporation into glucose (released by acid hydrolysis), lignin (Feely and Wislocki, 1991; Crouch and Feely, 1995) or fatty acids (Wislocki et al., 1989) occurs. The extractable residues from the present cabbage study involving emamectin benzoate are also complex (Crouch et al., 1997) and include sugars containing incorporated radioactivity (L. Allen et al., manuscript in preparation). Also, 12 separate avermectin degradates have been isolated from methanol rinses of cabbage treated with a single dose of emamectin benzoate at 0.3 lbs a.i./acre (Wrzesinski et al., 1996). These studies show that avermectins are attacked by various oxidative processes at different positions around the molecule, apparently producing fragments of the macrocycle that can be incorporated into natural products. However, no such fragments have been identified to date.

The current study of unextractable residues in cabbage also supports these observations. Radioactivity is

released from the M/W marcs (Table 2) by enzymes specific for cellulose, starch, and protein, and radioactivity is also removed by DMSO, which is known to solubilize lignin (Haque et al., 1976; Honeycutt and Adler, 1975). When the unextractable residue fractions (Figure 2) from the 5× plants were characterized by a protein assay, the protease hydrolysate accounted for nearly 57% of the protein (Table 3) and 23−26% of the radioactivity (Table 2) in the M/W marc; the DMSO fraction accounted for another 24% of the protein and 18−29% of the radioactivity. The protease may not have completely hydrolyzed the proteins in the pectinase marc to water-soluble peptides and amino acids during the protease digestion step, which immediately preceded the DMSO solubilization (Figure 2). Therefore, the DMSO could have possibly solubilized residual peptides present in the protease marc while also solubilizing the lignin in that marc. Also, proteins have been shown to be covalently bound to lignin (Whitmore, 1982). Therefore, protein fragments may have remained bound to lignin after the protease treatment.

When the unextractable residue fractions from the 5× plants were analyzed for glucose, a mean of about 72% of the released glucose (Table 4) and 9−12% of the released radioactivity (Table 2) was found in the cellulase hydrolysate. Another 9% of the released glucose was found in the amylase hydrolysate after treatment with maltase (Table 4). Furthermore, treatment of the amylase hydrolysate with maltase increased the glucose content of the amylase hydrolysate (not shown) as expected since the maltose released from the cellulase marc by amylase would be further hydrolyzed to glucose.

Pectinase released more glucose than any other enzyme except cellulase, accounting for approximately 16% of the total released glucose (Table 4). It was later determined that the pectinase used in the present study contained contaminating cellulase activity (Wrzesinski, C. L., unpublished results). Therefore, it was not clear how much of the radioactivity released by pectinase could be attributed to pectin.

A large part of the extractable residues from lettuce (Crouch and Feely, 1995) and cabbage (Crouch et al., 1997) consisted of a polar fraction of great complexity. Characterization of the polar fraction indicates that these residues are not conjugates of MAB1A. Also, some polar residues from cabbage, lettuce, and corn have been shown by HPLC to be simple sugars containing incorporated radioactivity (L. Allen et al., manuscript in preparation). Sugars are an important source of many plant biomolecules. The presence of soluble sugars containing incorporated radioactivity suggests that incorporation of radioactivity into other types of cabbage natural products such as cellulose, protein, lignin, and starch could occur. The release of radioactivity, especially by enzymes specific for distinct natural products, in each of the steps listed in this report supports this theory. Furthermore, some of the radioactivity in the M/W-extractable residue may be incorporated into proteins. For example, approximately 30% of the protein and/or peptides of the cabbage from the 5× cabbage plants was found in the extractable residue (not shown).

The unextractable residues in lettuce treated with MAB1A most likely consisted partly of starch and cellulose containing incorporated radioactivity since [^{14}C]glucose was released after acid hydrolysis of M/W marcs (Crouch and Feely, 1995). Also, portions of unextractable residues from both lettuce (Crouch and Feely, 1995) and cabbage, in the current study, were solubilized with hot DMSO, indicating association of residue with lignin. The cabbage study suggested, for the first time, incorporation of radioactivity into proteins. A previous study of unextractable residues of avermectin B_{1a} in celery also showed incorporation of radioactivity into glucose and association with lignin (Feely and Wislocki, 1991).

The results of this study indicate that the unextractable residues of emamectin benzoate in cabbage are probably natural products containing incorporated radioactivity and are therefore not of toxicological concern. Only about 1% of the initial recovered radioactivity in the marcs remained uncharacterized after using the procedure outlined here. Furthermore, the observed loss of total residue with time may result from metabolic turnover of these natural products. Studies to address the nature of incorporation of MAB1A fragments into natural products are in progress.

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